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Review

A Median Third Eye: Pineal Gland Retraces Evolution of Vertebrate Photoreceptive Organs†

Hiroaki Mano and Yoshitaka Fukada*

Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Japan

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ABSTRACT

In many vertebrates, the pineal gland serves as a photoreceptive neuroendocrine organ. Morphological and functional similarities between the pineal and retinal photoreceptor cells indicate their close evolutionary relationship, and hence the comparative studies on the pineal gland and the retina are the keys to deciphering the evolutionary traces of the vertebrate photoreceptive organs. Several studies have suggested common genetic and molecular mechanisms responsible for their similarities, but largely unknown are those underlying pineal-specific development and physiological functions. Recent studies have identified several *cis*-acting DNA elements that participate in transcriptional control of the pineal-specific genes. Genetic approaches in the zebrafish have also contributed to elucidating the genetic network regulating the pineal development and neurogenesis. These efforts toward elucidating the molecular instrumentation intrinsic to the pineal gland, back to back with those to the retina, should lead to a comprehensive understanding of the evolutionary history of the vertebrate photoreceptive structures. This article summarizes the current status of research on these topics.

INTRODUCTION

The evolutionary processes for the structure/function of various organs in living organisms have been of great interest in molecular, developmental and evolutionary biology. Among these organs, the eyes have attracted particular attention owing to their structural varieties albeit all serving to detect light. A promising approach to cutting this Gordian knot in biology is a comparative analysis of molecular characteristics of the eyes among species. Indeed, a series of genetic studies have demonstrated that a highly conserved transcription factor Pax6 serves as a master control gene for the eye morphogenesis in both vertebrate and invertebrate species (1). This finding strongly supports the idea that various types of the eyes found in the animal kingdom have a common evolutionary origin, in spite of their significant anatomical dissimilarities. A comparison between the eyes of phylogenetically distant animals, for instance, between the vertebrate camera eye and the insect compound eye, is helpful to reconstruct a hypothetical primitive structure of their common ancestral eye that could have emerged at a very early stage of the evolution. On the other hand, such a comparison provides little information about how the primitive eye has evolved into the modern sophisticated eyes present in various animal taxa.

An insightful inspection of these processes could be accomplished by a comparative analysis between the eyes or eye-like structures that exhibit distinct but sufficiently comparable designs with each other. The extra-ocular photoreceptive structures present in various vertebrate species (2) offer an unparalleled opportunity to explore the unique evolution and diversification of the photoreceptive structures including the camera eye. The most prominent and well-developed extra-ocular photoreceptor is the pineal gland (also referred to as the pineal organ or the epiphysis), an organ that has developed only among vertebrates and retains photoreceptive function in many nonmammalian species. Molecular and phenotypic evidence indicates a close evolutionary relationship between the pineal gland and the neural retina of the eye, particularly within their photoreceptor cell lines. The photosensitive pineal gland of some vertebrate species has a considerably simpler structure that possibly reflects an ancient form of the vertebrate retina, providing an excellent model for comparative developmental studies.

In this review, we focus on recent progress in understanding genetic variation of the pineal photoreceptor cells, regulation of pineal-specific gene expression and development of the pineal gland. On the basis of these molecular data, we discuss a potential application of a cell-type homology approach to the comparative evolutionary studies between the pineal gland and the retina.

INTERSPECIFIC VARIATION OF THE PINEAL GLAND

Similar to the optic vesicle, the pineal gland evaginates from the roof of the diencephalon during development (3). The pineal gland in the adult brain is a midline structure that

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*Corresponding author email: sfukada@mail.ecc.u-tokyo.ac.jp (Yoshitaka Fukada)

exhibits highly variable shapes among vertebrate species, and is often associated with an accessory organ such as the parapineal organ in lampreys and fishes, the frontal organ in amphibians, and the parietal eye in lacertilian reptiles (3). A well-conserved physiological function of the pineal gland is to rhythmically produce melatonin, an indoleamine hormone that is involved in regulation of biological rhythms (4,5). In mammals, the pineal gland is no longer photosensitive but serves as a neuroendocrine organ, in which the activity of melatonin synthesis is regulated by external neural inputs from the sympathetic nerves. In contrast to the mammalian counterpart, the pineal gland of the other vertebrate classes, such as lampreys, fishes, amphibians, reptiles and birds generally retains photosensitivity, and plays an active role as the photosensory organ in some species. The pineal gland of several animals also contains an intrinsic circadian oscillator, which is entrained to environmental light–dark cycles due to the endogenous photic input pathway (4). These three components of the clock system, *i.e.* the circadian oscillator, the photic input and the melatonin output machineries, all co-exist in individual pineal cells of the chicken and possibly in those of some other vertebrate species (6–8).

The functional transformation of the pineal gland from the photosensory to neuroendocrine organ is closely correlated with changes in its cellular composition and cell morphology (4,9). In lampreys, fishes and amphibians, the pineal photoreceptor cells are endowed with well-developed lamellar outer segments that closely resemble those of their retinal photoreceptor cells. The light signals captured in the pineal photoreceptor cells are not only transduced into intracellular signals regulating melatonin production, but also transmitted to secondary neurons through synaptic contacts within the pineal gland. These secondary afferent neurons, also called the projection neurons, innervate several areas of the central brain, similar to the retinal ganglion cells (10,11). The pineal photoreceptive cells of reptiles and birds are often called modified photoreceptor cells, which possess regressed outer segments with the lamellar structures degenerated to various degrees. The secondary neurons in the pineal gland are less abundant among these species, possibly as a consequence of reduced photosensory function requiring afferent outputs. Finally, the light-insensitive pinealocytes in the mature mammalian pineal gland lack pronounced outer segment-like structures, although they are regarded as a cellular homolog of the pineal photoreceptor cells of the other vertebrates.

The phylogenetic variation in the photoreceptor-related cells of the pineal gland is generally interpreted as a gradual transformation that has occurred in a single cell lineage during the evolution of vertebrates (Fig. 1a) (4). However, Ekström and Meissl (9) recently proposed a new hypothesis that the pineal gland of vertebrates commonly harbors the repertoire of photoreceptor-related cells, and the observed interspecies variation of the cellular composition should originate from ontogenetic changes in cell-fate restriction from progenitor cells during the evolution of vertebrates (Fig. 1b). These two hypotheses are not mutually exclusive, and relative contribution of the two mechanisms remains to be elucidated by further detailed studies, in particular, on the molecular and developmental features of various pineal cell types.

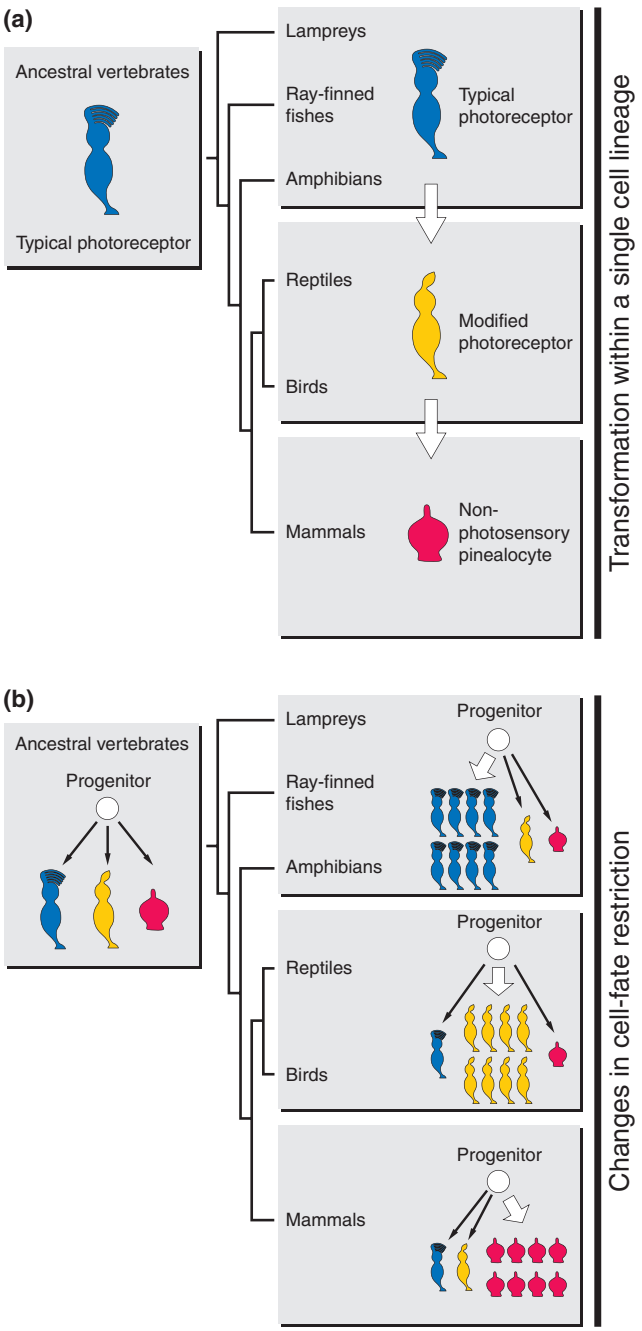

Figure 1. Two hypotheses accounting for the dynamic evolutionary changes of vertebrate pineal cells. (a) Successive regression of photoreceptor phenotype within a single cell lineage. (b) Changes in cell-fate restriction from a common progenitor during development.

MOLECULAR SIMILARITY AND DIVERSITY BETWEEN PINEAL AND RETINAL PHOTORECEPTOR CELLS

In addition to the morphological similarities, the molecular kinship between the pineal and retinal photoreceptor cells underpins their close evolutionary relationship. The pineal cells of the chicken and fishes contain a series of components found in the retinal phototransduction pathway, such as

opsins, transducin (12–14), cGMP-phosphodiesterase (15), cGMP-gated cation channel (16) and interphotoreceptor retinoid-binding protein (14,17). These observations suggest that the pineal photoreceptor cells share a similar phototransduction mechanism with the retinal photoreceptor cells (18). Interestingly, expression of several phototransduction genes is retained in the light-insensitive mammalian pinealocytes (19). This fact supports the idea that the mammalian pinealocytes have evolved from photoreceptor cells (Fig. 1), although the physiological relevance of the remnant expression of these genes remains unclear.

In contrast to these genes expressed in both the pineal and the retinal photoreceptor cells, a subset of genes that are expressed selectively in either of the two organs reflect their independent evolutionary paths. Chicken pinopsin with a blue-light sensitivity was the first example of pineal-specific opsin that is closely related to but distant from the retinal opsins (20,21), and pinopsin has a chimeric biochemical property between the rod and cone visual pigments (22). In the chicken, pineal opsin(s) is functionally coupled with two different types of G-proteins, G_{t1} (13) and G_{11} (23), which mediate acute inhibition of melatonin synthesis and entrainment of the intrinsic circadian oscillator, respectively. The pineal expression of pinopsin gene has also been found in other birds (24,25) and reptiles (25,26), but is very weak or undetectable in the other vertebrate classes (25). Another variant form of the pineal-specific opsin is exo-rhodopsin (Exorh) that was originally identified in the pineal gland of the zebrafish (27). This animal is unique in that it possesses two duplicated rhodopsin genes, *exorh* and retinal *rhodopsin*, which are expressed selectively in the pineal photoreceptor cells and the retinal rods, respectively (27). Phylogenetic analyses indicated the occurrence of *exorh* gene early in the ray-finned fish lineage (27,28), and pineal-specific expression of an *exorh* ortholog (extra-retinal rod-like opsin) has also been demonstrated in the Atlantic salmon (28). These observations, together with the fact that the rhodopsin-like immunoreactivities have been detected in the pineal gland of various teleost species (9,29,30), strongly suggest that teleosts generally have exo-rhodopsin gene that is expressed in a pineal-specific manner. In contrast to these pineal-specific opsins, several opsins such as red (20,26,27,31), green (26,32), blue (26) and ultraviolet (26,32) cone opsins are detected in both the pineal gland and the retina. Expression of the multiple opsin genes within a single pineal gland is consistent with immunohistochemical studies demonstrating heterogeneity of the pineal photoreceptor cells in terms of the opsin distribution (9,24,33,34), while the majority of the pineal photoreceptor cells of the chicken (24) and the zebrafish (27) express pineal-specific opsins.

Recent studies also identified novel members of non-visual type opsins possessing more peculiar features in the pineal gland of some vertebrate species. The lamprey pineal gland and parapineal organ express parapinopsin, which shows a reversible photoreaction with its stable photoproduct, an interesting feature common to those of invertebrate rhodopsins (35). In the chicken, both the pineal gland and the retina express melanopsin (36,37), which is remarkably distant from the vertebrate canonical opsins in molecular phylogeny, possibly suggesting an additional aspect of their molecular similarity.

REGULATION OF PINEAL-SPECIFIC GENE EXPRESSION

Tissue- and cell type-specific control of gene expression is expected to play a pivotal role in generating the molecular basis for physiological characteristics that are similar or different between the pineal and retinal photoreceptor cells. One of the key regulators responsible for both pineal and retinal photoreceptor cell-specific gene expression is cone rod homeobox (Crx), an Otx-related homeodomain transcription factor that binds to bicoid-type TAATCC recognition sequences (38,39). In the retina, Crx transactivates photoreceptor cell-specific genes including opsin genes, and regulates photoreceptor cell differentiation (38–40). Both genetic (40) and *in vitro* (41) studies in rodents demonstrated that Crx also participates in the transcriptional control of several pineal (-specific) genes such as *arylalkylamine N-acetyl transferase* (*aanat*). Similarly, circadian gene expression in the zebrafish pineal gland requires Otx5, which is closely related to Crx (17). These findings suggest that Crx/Otx5 serves as a common genetic instruction that contributes to the overall similarity between the pineal and retinal photoreceptor cells.

Although little is known about the molecular mechanisms directing specificity difference between the pineal gland and the retina, recent studies identified several *cis*-acting DNA elements that are involved in regulation of pineal-specific gene expression. A detailed mutation analysis of the zebrafish *exorh* promoter demonstrated that the pineal-specific activity of this promoter requires not only putative Crx/Otx binding sites, but also a 12-bp sequence (TGACCCCAATCT) designated PIPE, an abbreviation of pineal expression-promoting element (42). Chimeric rhodopsin promoters appended with the PIPE sequence in a foreign position can induce the gene expression in the pineal gland in addition to the retinal photoreceptor cells. These findings suggest that Otx5 and uncharacterized PIPE-binding protein(s) constitute a combinatorial code for the pineal photoreceptor cell-specificity.

Another pineal gene, zebrafish *aanat2* (43) appears to be transactivated synergistically by a combinatorial action of Otx5 and BMAL/CLOCK heterodimer via PRDM, an abbreviation of pineal-restrictive downstream module that comprises both Crx/Otx-binding sites and a functional CACGTG E-box for BMAL/CLOCK-binding (44–46). Because no circadian variation is observed for *otx5* mRNA levels in the zebrafish pineal gland, circadian regulated genes including *aanat2* is likely under the coordinated control of Otx5 and temporal regulatory factors such as BMAL/CLOCK heterodimer.

Another important regulator for the pineal gene expression is environmental light signal, which is transduced intracellularly and eventually inputs to a DNA regulatory sequence, a light-responsive element abbreviated as LRE. A 18-bp sequence (TGGCACGTGGGGTTCCTC) present in the promoter of chicken pinopsin gene mediates apparently light-stimulated expression and hence it represents LREs, but interestingly, the sequence contributes to transcriptional repression in the dark (47,48). Similar to the zebrafish PRDM, the pinopsin LRE comprises a CACGTG E-box (underlined in the 18-bp sequence) that constitutes the core sequence required for the light-dependent gene regulation (48). The expression

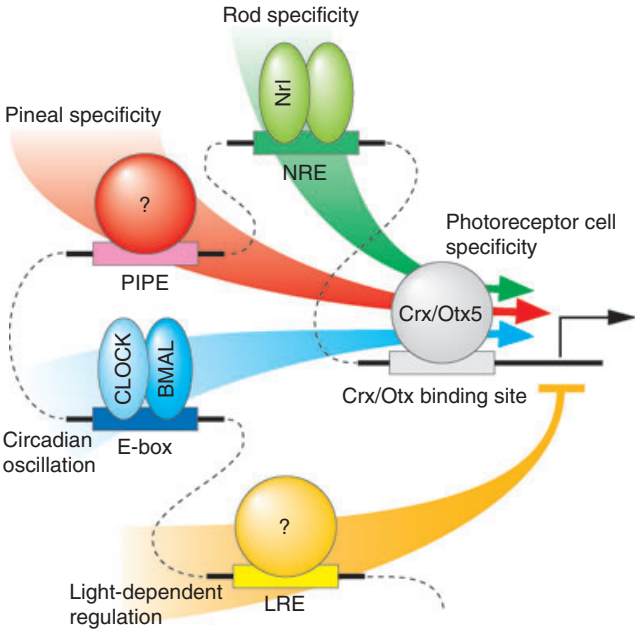

Figure 2. Cooperation of multiple *cis*-acting elements and transcription factors for regulation of photoreceptor cell-specific gene expression. This scheme illustrates an upstream region of hypothetical gene possessing all the *cis*-acting elements found in different genes and animals. Neural-retina leucine zipper (Nrl) is a candidate of the transcription factor that specifies rod photoreceptor cells in the retina (78,79). Nrl binds to Nrl responsive element (NRE) (80) and synergistically transactivates the rhodopsin promoter with Crx (39,81).

level of pinopsin gene exhibits daily fluctuation in the light–dark cycles but is constant in continuous dark condition (47), indicating the circadian clock-independent regulation despite the presence of the E-box in the LRE sequence. Therefore, the apparently conserved E-box elements in the pinopsin LRE and *aanat2* PRDM seem to function in manners quite different from each other. To sum up, the expression of pineal-specific genes could be regulated by orchestration of multiple *cis*-acting DNA elements and transcription factors that integrate spatial, temporal and environmental information into the expression levels of target genes (Fig. 2).

DEVELOPMENT OF THE PINEAL GLAND

Investigation of the ontogenetic development of the pineal gland would provide valuable insight into evolutionary processes of the vertebrate photoreceptive organs. The molecular mechanism for the pineal development has been best studied in the zebrafish by genetic approaches (Fig. 3). Floating head (Flh) is a homeodomain transcription factor that serves as a key regulator of the zebrafish pineal development (10,49). From the early stages, expression of *flh* can be detected in the prospective pineal gland, and seems to be controlled by diffusible factors that provide positional information. Indeed, *flh* expression is extended anteriorly by the *masterblind* (*mbl*) mutation (10), which inactivates Axin1, a negative regulator of Wnt signaling pathway (50,51). Similarly, *flh* expression expands more ventrally in *swirl/bmp2b* mutant embryos (52). In the downstream, Flh triggers sequential activation of transcription factors that probably participate in the pineal development, and *flh* mutant embryos consistently exhibit severe reduction of the neurogenesis in the pineal gland (10). Two basic helix-loop-helix (bHLH) transcription factors, Ash1a and Neurogenin1 (Ngn1), are located downstream of *flh* in the pineal neurogenesis pathway (Fig. 3) and play partially redundant roles in activating expression of further downstream genes, including *neuroD*, *islet1* (*isl1*), *otx5* and *onecut* (53). In parallel, expression of a T-box transcription factor Tbx-C is regulated by Flh but is not affected by disruption of Ash1a/Ngn1 function (53). Although the role of Tbx-C in the pineal development has not been determined, these observations suggest multiple independent pathways downstream of *flh* (Fig. 3). In the pineal gland of *mindbomb* (*mib*) mutant (54), *islet1*-expressing neurons (55) including *aanat2*-expressing neurons (43) increase in number, suggesting that the pineal neurogenesis involves Notch-Delta signaling pathway that is known to play a general role in the regulation of cellular differentiation.

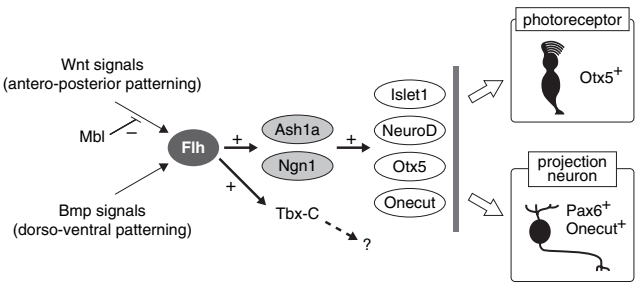

Figure 3. A regulatory network of genes controlling development and neurogenesis of the zebrafish pineal gland.

In contrast to these advances in understanding early pineal development, the mechanisms specifying the photoreceptor cells and the projection neurons remain largely unknown (Fig. 3). Ash1a and Ngn1, as well as Flh, are unlikely to regulate these specifications, because they affect the generation of both types of the neurons (10,53). In the zebrafish, Otx5 is a candidate for the specification of pineal photoreceptor cells, as judged by its requirement for maintaining the expression of many pineal genes (17). However, the activity of Otx5 alone does not fully account for the photoreceptor cell-specification, because Otx5 is not required for expression of several pineal genes, including *otx5* itself. In the mouse, conditional gene targeting of Otx2, another member of the Otx family, causes a total lack of both the retinal photoreceptor cells and the pinealocytes (56). It was also demonstrated that Otx2 can directly transactivate Crx expression (56). These findings revealed that Otx2 acts upstream of Crx in photoreceptor cell-fate specification, and a similar mechanism might operate in the zebrafish pineal neurogenesis. On the other hand, the projection neurons, which are located laterally in the zebrafish pineal gland (10), express selectively Pax6 (10) and Onecut (53,57). These homeodomain transcription factors are possibly involved in the specification of the pineal projection neurons in a way similar to the retinal neurogenesis, in which homeodomain transcription factors including Pax6 contribute to the neuronal subtype specification (58,59). In the retina, cell-fate specification of each neuronal subtype further requires a proper combination of bHLH-type and homeodomain-type transcription factors (59). For instance, a combination of bHLH factors Mash1/Math3 and a homeodomain factor

Chx10 directs specification of bipolar cells (60), while amacrine cell-fate specification requires a different combination of transcription factors, bHLH-type NeuroD/Math3 and homeodomain-type Pax6/Six3 (58). It is therefore likely that particular combinations of bHLH and homeodomain transcription factors control the cell-fate specification in the pineal neurogenesis as well.

EVOLUTION OF VERTEBRATE PHOTORECEPTIVE ORGANS

Diversification of neuronal subtypes should have been a critical factor that defines the evolution of the retina. The existing vertebrate retina is composed of six classes of neurons organized into three cellular layers. Among the retinal neurons, rod and cone photoreceptor cells are generally thought to share a common evolutionary origin, a ciliary photoreceptor precursor, and hence these two classes are sometimes combined into a single one. On the other hand, the origins of the other retinal neurons, *i.e.* bipolar, horizontal, amacrine and ganglion cells remain obscure. A recent attempt to address retinal cell-type homologies within and among animal species led to the hypothesis that the ganglion, amacrine and horizontal cells in the vertebrate retina could be traced back to a common evolutionary precursor (Fig. 4) (61). This hypothesis is supported by a number of similarities of molecules that operate in their developmental processes and physiological functions (61). For example, expression of Pax6 at the later developmental stage is detected in the ganglion, amacrine and horizontal cells but not in the rod, cone and bipolar cells of the vertebrate retina (61,62). More interestingly, the former three neurons probably share a common origin with rhabdomeric photoreceptor cells found in invertebrate retinas (61). This evolutionary scenario implies that the vertebrate retina is composed of at least two types of evolutionary distant neuronal classes, the ciliary and rhabdomeric cells (Fig. 4), and in fact it appears that these two types of photoreceptor cells had already coexisted in the common ancestor of the deuterostomes and the protostomes (63). A rhabdomeric type opsin, melanopsin, is expressed in the ganglion, amacrine and horizontal cells of some vertebrate species (64–68), providing a supportive evidence for their evolutionary kinship.

The cell-type analysis described above for the retinal neurons could be applied to the pineal neurons, and such a comparative point of view should provide valuable clues to further understanding of the evolutionary processes of the vertebrate photoreceptive organs. Striking similarities between the pineal and retinal photoreceptor cells indicate that they have diverged from a common precursor of the ciliary cell type. Even in the highly transformed state of the mammalian pineal gland, the pinealocyte represents characteristics of the ciliary type retinal photoreceptor cell such that they both produce melatonin (69–71), express a subset of genes for phototransduction components (19), and share developmental regulation depending on Crx/Otx-related transcription factors (40,56). On the other hand, the pineal projection neuron possibly belongs to the rhabdomeric cell type, because it expresses Pax6 at the later stage of the neuronal differentiation just like the retinal ganglion, horizontal and amacrine cells do (Fig. 3) (10). This idea may be supported by the functional similarity between the pineal projection neuron and the retinal ganglion cell, both of which project their axons to the central areas of the brain (10,11). Such parallelism delineates a fundamental composition of the cell lineages common to the pineal gland and the retina, raising the possibility that a common ancestor of the two organs had already consisted of two distantly related cellular components, ciliary and rhabdomeric precursors (Fig. 4). One of the open questions in the cell-type homologies is the enigmatic origin of the retinal bipolar cells (61), which relay neural signals from the photoreceptor cells to the ganglion cells (Fig. 4). Because recruitment of the bipolar cells appears to be a key evolutionary step in order for the vertebrate retina to establish the highly ordered structure of the three-layered neural network, it is worthwhile to unravel

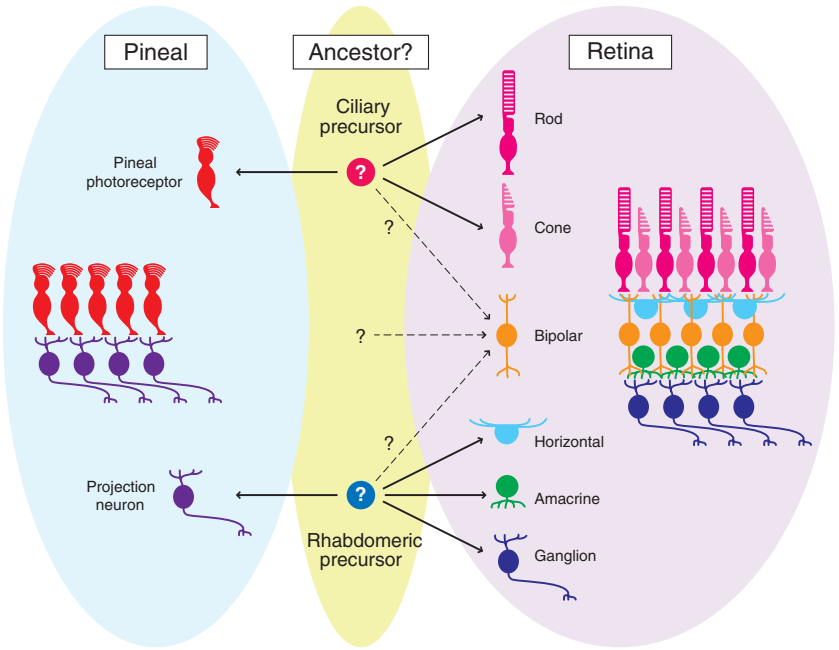

Figure 4. Parallelism of neuronal subtypes between the two typical photoreceptive organs in vertebrates, the retina and the pineal gland.

the origin of the bipolar cells by comparative developmental studies between the retina and the pineal gland, the latter of which lacks a recognizable cellular counterpart to the bipolar cells. At present, only limited information is available as to the molecular characteristics of the pineal cells, preventing a more detailed comparison of the cell-type homologies between the pineal and retinal neurons. Validation and refinement of the evolutionary model require further elucidation of the molecular mechanisms underlying the pineal-specific gene expression, developmental process and physiological functions.

Finally, understanding the mechanism for the pineal neurogenesis, in which both the ciliary and rhabdomeric type cells are generated from the same progenitor cells in a relatively simple manner (Fig. 3), would shed light on the evolution of the developmental processes that integrated the two distantly related cell-types into a single photoreceptive organ. Important clues to the issue may also be obtained by comparative studies on more primitive photoreceptor systems found in cephalochordates (72) and in the deep brain of some vertebrate species (73–76). The analyses of the cell-type homologies and the developmental mechanisms of these systems will ultimately provide a comprehensive and detailed view of the evolution and diversification of the vertebrate photoreceptive structures.

CONCLUDING REMARKS

The molecular analysis of the pineal gland provides a unique opportunity to investigate the evolutionary history of vertebrate photoreceptive organs, which has long attracted great enthusiasm of biologists since Darwin's time. The molecular mechanisms for the pineal development and cell-type specification are just beginning to be elucidated, and considerable progress will certainly be made in the near future. In addition to the molecular comparison among the retinal, pineal and other primitive photoreceptors, comparative studies of the pineal gland itself among species will provide helpful hints as to the mechanisms underlying dynamic evolution of the brain functions in vertebrates. In the zebrafish, the pineal complex has also been studied as a model for the formation of left-right asymmetry in the brain (77). These recent studies represent a trend of pineal research beyond the traditional way of analysis on the circadian clock and photoendocrine mechanisms.

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